THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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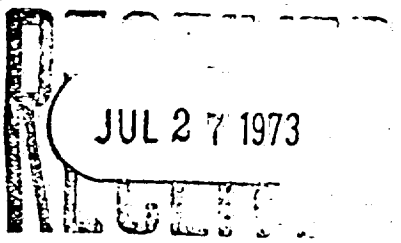

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal [X] Second Renewal [ ]

Date: July 26, 1973

1. Principal Investigator (give title and degrees): Walter B. Essman, Ph. D., M.D.
Professor of Psychology & Biochemistry

2. Institution & address: Queens College of the City University of New York
65-30 Kissena Boulevard
Flushing, New York 11367
and
The Research Foundation of the City University of New York
1411 Broadway, New York, New York 10018

3. Department(s) where research will be done or collaboration provided:

Department of Psychology, Queens College of the City University of New York

4. Short title of study:

"Metabolic Response To Stress--Tobacco Smoke Interactions"

5. Proposed renewal date: January 1, 1974

6. How results to date have changed earlier specific research aims:

The initial aims set forth in this proposal have been essentially unchanged by any of the results obtained to date. It has become clear, however, that several additional sources of data have become available through the application of alternate methodologies to materials obtained from several of the experimental protocols outlined.

7. How results to date have changed earlier working hypothesis:

Results to date have not altered earlier working hypotheses; the data have, in fact, offered considerable support to the general theme of the project, indicating that at several levels of cellular regulation, an interaction can, in fact, be demonstrated between several varieties of behavioral and/or physiological stress and the effects of nicotine.

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8. Any additional facilities now required? Describe briefly:

NONE

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Mr. Eliahu Heldman has been replaced by Mrs. Barbara Kornreich as a Grant Researcher on this project.

10. Append outline of experimental protocol for ensuing year.
11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See attached list.

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12. Summary progress report (append in standard form as separate document, unless recently submitted).

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13. Budget for the coming year:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Walter B. Essman, M.D., Ph.D. Principal Investigator, (summer 2/9ths)	100	$ 7,662. *
Barbara Kornreich	100	$12,700.
Fringe Benefits for B. Kornreich = 16%		2,032.
Technical		
Technical Assistant (to be selected)	100	$ 8,300.
Nancy Mulligan (Secretary)	100	7,900.
Fringe Benefits (16%)		2,592.
	Sub-Total for A	$41,186.
B. Consumable supplies (by major categories)		
Chemcials		$ 950.
Radiochemicals		1,850.
Centrifuge tubes, rotors, supplies		2,300.
Gases		400.
Glassware		800.
Animals		750.
	Sub-Total for B	$ 7,050.
C. Other expenses (itemize) (Travel)		
7th Winter Conference for Brain Research, Vail, Colorado, Jan. '74		
International Congress of Physiological Sciences, New Delhi, India, Oct. '74		
American Physiological Society, New Jersey		
Collegium Internationale, Neuropharmacology, Paris, France, May '74	Sub-Total for C	$ 1,838.
Local Travel Expense		
	Running Total of A + B + C	$50,074
D. Permanent equipment (itemize)		
Automatic Dishwasher		$ 750.
Beckman Centrifuge Roter		1,035.
	Sub-Total for D	$ 1,785.
E. Indirect costs (15% of A+B+C)	E	$ 7,511.
* This figure includes salary + fringe benefits.	Total request	$ 59,370.

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Studies of Nicotine Action on Memory Consolidation"	Council for Tobacco Research U.S.A.	$21,579.	January, 1973 to December, 1973

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Studies of Nicotine Action on Memory Condolidation"	Council for Tobacco Research, U.S.A.		January, 1974 to December, 1974

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Walter B. Essman

Signature Walter B. Essman Date July 26, 1973

Telephone 212 762-5949 ---
Area Code Number Extension

Checks payable to

Research Foundation

Mailing address for check:

1411 Broadway

New York, N.Y. 10011

Responsible officer of institution

Typed Name 1) Hannah Petzenbaum
2) Albert M. Levenson

Title 1) Research Foundation
2) Assoc. Dean of Faculty - Queens College

Signature Hannah Petzenbaum Date 7/26/73

Telephone 212 445-5275
Area Code Number Extension

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11. PUBLICATIONS

1. Essman, W.B. Neurochemical changes in ECS and ECT. Seminars in Psychiatry, 1972, 4, 67-70.

2. Essman, W.B. Drug effects and learning and memory processes. In: Garattini, S., and Shore, P. (Eds.). Advances in Pharmacology and Chemotherapy. N.Y.: 8, 1971, Academic Press, Pp. 241-330.

3. Essman, W.B. Nicotine-related neurochemical changes: some implications for motivational mechanisms and differences. In: Dunn, W.J., (Ed.). Smoking Behavior, 1973, 281-283.

4. Essman, W.B. Effects of ECS on cerebral protein synthesis. In: Fink, M., Kety, S.S., McGaugh, J., and Williams, T. (Eds.). The Psychobiology of ECT. Washington, D.C., V.H. Winston & Sons, 1973.

5. Essman, W.B. Regional alterations of synaptic O-phosphorylethanolamine in differentially housed mice. Rass. Clin. Scient., 1973.

6. Essman, W.B. Effetti dell'elettroshock sulla neurochimica del sistema nervaso centrale, I. Rass. Clin. Scient., 1972, 48: 361-370.

7. Essman, W.B. Effetti dell'elettroshock sulla neurochimica del sistema nervaso centrale, II. Rass. Clin. Scient., 1973, 49: 5-23.

8. Essman, W.B. Tissue distribution and central effects of digoxin in mice: effects of an acute and chronic stress. Pharm. Res. Commun., 1973 (In Press).

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Brief Description of Objectives or Specific Aims:

With the development of several methods in cellular biology, molecular biology, and neurobiology, it has become increasingly more possible to evolve cellular and subcellular model systems within which aspects of such processes as development, aging, and pathology may be studied; as a direct consequence of being able to utilize simplified model systems of this type condiserable economy of technology, theory, and generation of testable hypotheses has been achieved. One purpose in the organization of this general research program is to investigate, within the context of such model systems, the effects of acute and chronic stress; the basis for the selection of the model systems within which stress effects would be investigated resides within, generally, a distinction between peripheral and central nervous system models and measures, and these will be utilized as a descriptive and predictive means of assessing acute and chronic stress effects, in terms of onset, duration, and adaptation. Within this same context it will be the purpose of the proposed investigation to view the interaction of acute and chronic stress effects upon cellular model systems and the acute and chronic treatment with nicotine, the latter being considered within the context of a pharmacological event.

The proposed project is directed toward the evaluation of the nature and sequence of metabolic and biochemical changes resulting from stress and/or its systems showing adaptation to stress and their interaction with the effects produced by nicotine. The basis of the objectives of this proposed work is a definition of those cellular and subcellular units, the biochemical or metabolic composition of which, may be altered by either stress, nicotine treatment, or the interaction of the two. The indices of stress and the interactive effects thereupon contributed by nicotine treatment will reside within a consideration of biologically active molecules which characterize a cellular or organelle system with which they are classically identified. As such, changes in the content, storage pool levels, or

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turnover of these molecules may well provide extremely sensitive indices of how stress and nicotine treatment interact, and the nature by which the individual or interactive contributions of both provide meaningful hypotheses concerned with the development of pathology related to processes within such cellular or organ systems, as well as being descriptive of the more basic mechanisms responsive to such biological agents.

Brief Statement of Working Hypothesis:

The functional basis upon which the proposed research project rests is the assumption that cellular mechanisms as models for the effects of acute and chronic stress will serve as a meaningful basis for assessing the interaction of such stress responses and treatment with nicotine by experimental means. In view of the strong evidence for the prevention, antagonism, or reordering of site-specific mitochondrial metabolism produced by stress, through inhalation of gaseous phases from nicotine treatment (Riesen & Kyle, 1969) both the feasibility of an organelle model system for stress - nicotine treatment, deriving from such sources as liver and lung tissue, and the feasibility of investigating other cellular and subcellular systems, have been strongly indicated. It is not the purpose of this research to explore the multi-faceted systems within which stress operates or nicotine treatment exerts measurable effects, but to focus primarily upon five tissue systems from which the cellular and subcellular biochemical data may be derived in terms of those molecules which appear to be most significant for the regulation of physiological and pathophysiological processes within such organ systems. Those general hypotheses which are generated both on the basis of research findings from our laboratory, as well as from other work relating to the primary goal of this project, are that (1) models of acute and chronic stress, respectively may be developed and may reflect, in responses observed for several organ systems, changes

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that are coincident with the onset, duration, and adaptation to stress; (2) several of those changes which are utilized as indices of stress onset, permanance, or reversibility may be interacted upon by the effects of nicotine; (3) the nature of the biochemical or metabolic change observed within representative cellular model systems in response to either stress, nicotine, or their combination will be utilized as predictors of the onset of ensuing breakdown of functional metabolic systems; (4) the objective role of nicotine which serves to either alter the time course for onset of stress indices or changes the sequence over which they may o-cur or be modified will be assessed.

Details of Experimental Design and Procedures:

A well documented series of physiological changes resulting from acute stress, utilizing a considerable variety of stressor agents and/or events has been presented (Selye, 1950). On the basis of the work summarized and directed experience with one reliable stressor which easily meets the experimental needs for such a condition, we intend to utilize restraint-stress in rodents (mice, rats and guinea pigs) as the basis for the acute stress condition. A number of related variables concerning this form of acute stress have been defined in a number of studies completed in our laboratory, and these include such factors as food intake, time of day, age, etc., all of which can be controlled to provide conditions wherein this acute stress can be defined in terms of: (a) duration, and (b) frequency. The conditions constituting chronic stress have further been well documented in a series of experiments originating in our laboratory between 1964 and 1971 and these are basically involved with the utilization of isolation housing which consittutes the basis for chronic stress, the magnitude of which and adaptation to which can be titrated by a single variable of the duration of which the rodent is maintained under isolation. The proposed work rests upon the investigation of both peripheral as well as central cellular changes which accompany or follow stress and the resistivity by

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which changes in interaction with nicotine occur. Specifically in peripheral tissue, those models of direct functional significance to be studied are as follows: (1) cardiac tissue (norepinephrine, acetylcholine); (2) adrenal tissue (epinephrine); (3) platelets (5-hydroxytryptamine); and (4) gastrointestinal tissue (5-hydroxytryptamine).

(1) There are a number of biologically active molecules which, through several mechanisms of action, serve to provide for either the elevation or reduction of selected metabolic substrates indicated above as specific for given tissue sites. These drugs will be utilized in experiments wherein a given biologically active molecule is either increased or decreased for physiologically availability at this site. Thereby, a tissue-specific, molecule-specific state may be imposed, by which the effects of stress and/or nicotine treatment may be assessed. It is specifically the purpose of this methodological approach to provide for increases or decreases in trophic and/or transmitter mediation of substrates specific to given storage granules in those tissues outlined above. Those substrates proposed for this use consist of (1) metaraminolbitartrate which displaced norepinephrine, inhibits its uptake by norepinephrine containing storage granules, and thereby increases norepinephrine availability and release. This drug would thereby appear appropriate to provide for increased norepinephrine availability and release in cardiac tissue. A dose by which this effect may be achieved is 15 mg/kg.

(2) α-methyl-ρ-tyrosine is a substance with which leads to the inhibition of tyrosine hydroxylase the rate limiting enzyme in the biological synthesis of dopamine and norepinephrine. A dose of 80-100 mg/kg of this compound will lead to tissue depletion of these catecholamines of about 60% within five hours and maintain reduced tissue levels for approximately 90 minutes. Such treatment would again be effective in reducing endogenous levels of cardiac tissue catecholamines, which would

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be reversibly inhibited.

tissue, those models of direct functional significance to be studied are as

(3) 6-Hydroxydopamine can be administered in successive doses of approximately 300 mg to achieve an irreversible denervation of catecholamine containing storage granules in cardiac tissue. Although there has been no previous data concerned with the use of this irreversible inhibitor its direct administration can achieve approximately 85-95% reduction in catecholamine storage by such tissue.

duration of (4) L-DOPA is the precursor of dopamine, which when administered peripherally, will account for increased central dopamine levels. Peripheral administration (200 mg/kg., i.p.) of dopamine will result in increased tissue uptake and conversion to norepinephrine to offer another alternative for increasing cardiac catecholamine levels.

(5) 6-Methoxytetrohydro-β-carboline is a β-carboline derivative, which following parenteral administration (5 mg/kg) will lead to highly significant increases in 5-hydroxytryptamine (5-HT), particularly in platelets, platelet-enriched plasma, and endochromaffin cells of the gastrointestinal tract. As such this compound provides an extremely potent pharmacological tool by which 5-HT, stored in these sites may be increased. Such an increase shows a peak effect by two hours following treatment with several hundred per cent increase being shown in these sites.

(6) 6-Hydroxy 5-Hydroxytryptamine is a recently synthesized substrate which, when administered parenterally, produces a marked and long lasting depletion of 5-HT concentration; the duration of this depletion is as long as 30 days, depending upon the dose utilized and the direct site of investigation. This compound will prove to be extremely useful in evaluating platelet or endochromaffin cell 5-HT depletion.

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(7) Oxytremorine is a substance specific to the cholinergic system, wherein its effect is to produce a significant elevation in tissue acetylcholine, the duration of which is dose-dependent. This substance may thereby be utilized to produce a reversible increase in cholinergic content of cardiac tissue, wherein its major effects following parenteral administration may be observed.

(8) Methyl Atropine is a compound which is specific to peripheral inhibition of post ganlionic cholinergic nerves and a blockade of the muscarinic effects of acetylcholine. There is considerable evidence to indicate that acetylcholine storage is modified by atropine-like compounds which lead to the depletion of this amine. It would therefore be appropriate to consider reduced cholinergic function brought about by this drug in cardiac tissue.

(1) Cardiac Tissue.

Catecholamines (epinephrine, norepinephrine) have been studied extensively in cardiac tissue. There seems to be a divergence of opinion as to whether or not the catecholamine containing structures are the cell bodies of this tissue or adrenergic nerve terminals. Catecholamine containing cells have been described (Jacobowitz, et. al., 1966), however these observations have not been confirmed by others (Dahlstrom, et. al., 1965). Within the neurons innervating cardiac tissue, there is evidence for different storage pools for norepinephrine. Specific microparticles (20-100mμ) have been isolated from cardiac tissue (Michaelson, et. al., 1964) in which a large fraction of the cardiac norepinephrine is bound. This apparently is represented in a partial fractionation of cardiac homogenates on the order of approximately 60-80% of the total cardiac content of this amine (Glassman, et. al., 1965). The presence of "free" norepinephrine apparently remains in considerable question. Histochemical evidence strongly suggesting the absence of any extraneuronal pool of this amine in cardiac tissue. Unfortunately this question

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has not been studied systematically and certainly it would appear particularly relevant to consider whether the presence of "free" cardiac norepinephrine can be utilized as an index of stress, inasmuch as changes in the total content of this catecholamine certainly have indicated (Selye, 1950). Therefore, it would seem quite reasonable to anticipate that conditions imposed upon the mechansims of synthesis, uptake, binding, or catabolism could easily affect the cardiac amine storage pools. With the availability of techniques for subcellular fractionation of cardiac tissue (Michaelson, et. al., 1964; von Euler & Lishajko, 1965) and separation, thereby, of storage pools from homogenized cardiac tissue, determinations will be made of "free", to "bound" ratio of this cardiac catecholamine in order to assess the quantative relationship between acute and chronic stress and the interaction therewith of nicotine treatment and changes in the rates of these storage pools. There are some bases for a consideration of cardiopathies which reside within observations, on one hand of cardiac catecholamine depletion in heart failure induced experimentally, or observed clinically (Chidsey, et. al., 1964; Chidsey, et. al., 1963; Chidsey, et. al., 1966) and increases in the cardiac liberation of norepinephrine associated with myocardial infarction (Kuschke & Schneider, 1960) and in cardiac decompensation (Kuschke, 1961). The production of focal myocarditis and hemeragic lesions localized to the pericardiam and endocardiam have been observed following injection of norepinephrine in dogs (Szakacs & Cannon, 1958), as well as in post mortum examination following therapeutic infusion of norepinephrine. An increase of liberation of norepinephrine from cardiac tissue has, as previously indicated, been well established in both experimental animal studies (Selye, 1950) as well as in man (Elmadjian, et. al., 1956; Elmadjian, et. al., 1957) and in addition to this, the interesting observation that the release of cardiac norepinephrine may also be accomplished by nicotine (Westfall & Watts, 1964). One pathophysiological consequence which has been demonstrated in cardiac tissue as a consequence of

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restraint stress in cardiac necrosis (Selye, 1950), and typical stress cardiopathy, consisting of disseminated micronecroses has been more extensively studied (Selye, 1961) and likened to cardiopathy as developed with isoprotemol and catecholamines. The biochemical commitants of such stress-induced cardiac pathology have not been documented nor has the change in the time course of onset as potentially indicated for nicotine treatment been studied within this context. It seems apparent, in view of the foregoing evidence, that acute and possibly chronic stress, and their potential interaction with nicotine would seem extremely likely as conditions for the modification of norepinephrine storage pools measured in cardiac tissue. Use, therefore, of such storage pool ratios will be made in assessing the onset, duration, recovery, and/or adaptation to acute stress (restraint) or chronic stress (isolation).

In as much as the vagal innervation of the heart is mediated by cholinergic endings in that tissue, an avenue for consideration of synapses maintained by acetylcholine is provided for study. Isolation of such cardiac synaptosomes by differential and density gradient ultracentrifugation will provide a basis for study of the synthesis, uptake, storage, release, and turnover occurring at cardiac cholinergic endings. The effects of stress (acute or chronic) upon cardiac cholinergic innervation and metabolism have not been studied, as have not the effects of nicotine upon this system; although, in the latter instance, the cholinergic effects of nicotine would warrant consideration of this potential source of interaction with stress at cholinergic endings in the heart.

The conditions under which stress-nicotine parameters will be regulated will consist of treating both conditions as independent variables within the context of a pharmacological paradigm, i.e., the duration of acute or chronic stress will be

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titrated against the duration and frequency of nicotine treatment. Furthermore, the sequence, as well as the interval between stress and nicotine treatment will be varied; i.e., stress either proceeding or following treatment at intervals ranging from 10 minutes to 24 hours, or if the emerging data so indicates, the two variables will be superimposed in time. Nicotine, as the alkaloid will be used in acute or chronic doses ranging from 0.1 - 0.8 mg/kg., i.p. As indicated above, for those studies concerned with one of the four peripheral systems to be investigated (cardiac tissue), the other three peripheral sources will be simultaneously investigated utilizing the same animals from which the cardiac tissue is obtained; i.e., adrenal tissue, platelets, and gastrointestinal tissue.

(2) Adrenal Tissue.

The relationships between adrenal morphology and chemistry and the concept of "emotionality", as well as consequences of stress, have been known for some time. Rats selectively bred for high levels of "emotionality" have been shown to have heavier adrenals (Yeakel & Rhoades, 1941) and among wild rats showing both greater "emotionality" levels as well as an increased degree of reactivity to environmental cues, heavier adrenal weights were also demonstrated (Rogers & Richter, 1948). The "reactivity" of adrenal tissue to stress has been clearly documented (Selye, 1950) and the nature of the storage and release mechanisms upon which the active constituents of this tissue depends has also been extensively studied (Blaschko, 1954). The nature of the catecholamine storage granules in adrenal tissue has presented an extremely interesting conceptual framework within which mechanisms operative both during and/or following stress may be further studied. The chromaffin granule and its catecholamine-binding protein, chomogranin, have offered considerable promise as mechanisms which provide further insight into those processes which affect the synthesis, storage, and release of adrenal catecholamines. The techniques for isolation of both the chromaffin granules, which are totally adrenergic in content, have been accurately worked out utilizing differential and density gradient

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centrifugation methods (Blaschko, et. al., 1955). It will be the object of the proposed investigation, in dealing with isolated chromaffin granules from rodent medulla, during and following both acute and chronic stress, to assess changes in catecholamine content and turnover. It has been shown that the effect of smoking upon the release of epinephrine appear dependent upon the duration of inhalation or dosage, as well as upon the interval and probably frequency thereof, intervening between successive treatments (Watts, 1960). The premise upon which observed changes in urinary excretion of catecholamines in man during smoking is based is that this reflects adrenal medullary secretion. There appears to be no systemic evidence relating the effects of nicotine, to specific changes in the storage granules in the adrenal tissue. It will be the purpose of this series of experiments to describe the interaction of stress and nicotine, investigated within the context of the previously outlined experimental paradigm, with a view toward defining this interaction on the basis of changes in catecholamine synthesis, storage, and release mechanisms which operate during these events in the chromaffin granules of the adrenal.

(3) Platelets.

The known and suspected role of platelets in the maintenance of homeostasis are well documented (Kowalski & Niewiarowski, 1967; Johnson, et. al., 1961; Mustard & Packham, 1970). Furthermore, the pathological states leading to infarcts involving platelet aggregation and adhesion are known (Mustard & Packham, 1970). Various stressor events presented to rodents, such as physical restraint or social deprivation (acute and chronic stress) produce a decrease in circulating levels of monodisperse platelets, presumably as a result of aggregation. In humans, various investigators have shown that cigarette smoking increases platelet adheseveness (Mustard & Murphy, 1968; Mustard & Murphy, 1963; Schievelbein & Werle, 1962).

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Presumably, the active constituent in cigarette smoke is nicotine (Werle & Schievebein, 1965). Little is known of the interaction of stress and nicotine on platelet physiology or biochemistry. Those tissue constituents, serotonin, norepinephrine, epinephrine and histamine, selected for investigation in this study are all capable of increasing platelet aggregation alone, or potentiating the action of one of the others (Mustard & Packham, 1970). However, in the case of serotonin, it has been shown that a tachyphalaxis develops; this furthermore produced tachyphalaxis to the aggregating action of epinephrine and ADP (Baumgartner & Born, 1968). These aggregating-inducing agents, which can act in vivo as well as in vitro, promote the release of certain platelet constituents which act to cause platelet lysis (Davey & Luscher, 1968; Holmsen, et. al., 1969). The constituents of interest to this study are histamine, catecholamines, serotonin and lysosomal enzymes.

The localization of the former agents in platelets is thought to represent a means by which the circulating levels of these vasoactive agents are kept low, until the enzymes for synthesis are not present (Shore, 1962). The paradoxy of this situation is that those agents, epinephrine, serotonin and histamine are released from other sites during stress and in an attempt to reduce circulating levels, platelet uptake occurs. As has been shown for serotonin, it is the uptake process which causes aggregation and release of platelet constituents. The uptake requires the utilization of ATP which produces ADP, which in turn is the causative factor in platelet aggregation. Furthermore, the uptake process is not particularly specific for serotonin, but also operable for other amines and provides thereby the basis for the false transmitter concept (Kopin, 1966). These observations provide the basis for the hypothesis that platelet serotonin (the major amine present) is released as part of the initial stress reaction. The released serotonin

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is free to exert its pharmacological actions and can be taken up at amine storage sites where it is not normally present.

Isolation of platelets will be accomplished by sedimentation and differential centrifugation, allowing for both platelet counts as well as the study, in vitro of these units; serotonin content, precursor uptake and catabolism will be considered during stress and with the conditions wherein nicotine is also utilized as an independent variable. Direct extraction and reading of serotonin from platelets permits rapid and relaible assay of extremely small quantities of this amine. The general experimental procedures to be followed parallet those described previously.

(4) Gastrointestinal Tissue.

The role of 5-hydroxytryptamine (serotonin) in the regulation of the mechanical action of the intestine appears to reside in its stimulation of receptors in the mucosal tissue, as well as by providing sensitization to endogenous choline. The release of serotonin from its gastrointestinal storage sites, has been accomplished by a variety of stimuli including stress. Preliminary investigation of the relationship between acute stress (restraint) and gastric ulcer production in mice has led to the implication of serotonin in this process (Essman, et. al., 1971). Histamine, pecuilar to its own storage granules in the gastrointestinal system (the mast cell) has also been implicated as being important for stress; as both a causative factor, as well as a potential correlate by way of enzyme systems in common with gastrointestinal serotonin. The basis upon which present consideration of these two important biologically active amines is considered relates to their potential value as site-specific molecules related to both stress and nicotine treatment and being further able to describe the interaction of these two events.

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In a comprehensive study concerned with the effects of both acute (restraint) and chronic stress (isolation) considerable data have been accumulated for inter-relating such variables as serotonin turnover, histamine content, gastric pH, etc., to the emergence of gastric tissue pathology. The relative contributions of these amines and their respective storage sites within the gastrointestinal system will be considered, specifically with reference to their role in response to stress as well as to nicotine treatment and the conditions under which these two events participate either temporally or spatially in the interaction with one another.

(2) Aside from those basic schema for acute and chronic stress specified above, several additional conditions will be imposed which have been shown to serve as effective models, particularly for somatic changes. These include (a) footshock, (b) electroconvulsive shock, and (c) sleep deprivation. The means by which these stressors are effected and the parameters utilized in their initiation have been worked out and lend themselves easily to use and adaptation in our laboratory.

The areas of primary attention toward which the above tissue systems will be directed will concern the relationship between "free" and"stored" pools of the specific tissue amine under consideration and the quantitative relationship between those tissues wherein multiple amine-regulated function is indicated. It is therefore our purpose to consider, on the basis of molecular ratios, those changes brought about by specific stressors in amine storage systems which provide for a release from storage in another amine system. An example illustrating this relationship may be found in adrenal tissue wherein adrenaline is released from chromaffin granules by the appropriate stimulation through nerve endings which release acetylcholine.

It is surprising, however, that these requirements for such adrenergic release have never been specified in terms of qualitative synaptic release of acetylcholine. Similar conditions prevail in cardiac tissue as well as, of course,

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in brain tissue.

The use of specific inhibitors and releasors for specific storage granules substrate system, as we have previously outlined, will serve as a basis for the isolation of such substrates from the specific tissue sites considered as a consequence of stress or in interaction with nicotine treatment. An interesting observation may be made in that most of those tissue and storage granule systems to which reference has been made, there are endogenous variations in both substrate levels as well as in ratios of "free" and "bound" substrates. This consideration will be given attention in several experiments specifically designed to consider cyclical variations in substrate content and storage and how this may provide a basis for differences in the effects of stress and/or nicotine treatment.

The means by which and rationale for isolation and selection of specific subcellular components from those indicated tissue sites under stress or in combination with nicotine treatment have been previously outlined; the significance of this approach lies in the ability to specify changes in the subcellular distribution of substrates, which by virtue of either stress and/or nicotine treatment may be changed through alterations in a "bound" to "free" disposition of a given substrate. This approach will also provide a source for relative purified subcellular components which can be isolated either following in vivo studies and separated for in vitro studies in order to investigate the potential difference in the response of such organelles originating from different cell sources to exogenous substrates comparable to those contained within these cellular storage sites.

(5) Stress-Nicotine Interactions and Central Nervous System Models.

In the central nervous system, it has been generally agreed that the most vulnerable component of change, pathological processes, insult, or phasic event

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is the synapse; the response of the synaptic region and the functions which these units serve will be investigated under both conditions of acute and chronic stress. Such consideration will involve a relationship of synaptic events to observe peripheral changes, considered on the basis of their onset, duration, and recovery with a view toward specifying some of the neurobiological consequences of the interaction of stress with nicotine treatment.

With the advantage of having available techniques for the subcellular isolation and characterization of pre-synaptic nerve endings (synaptosomes) from various regions of the mammalian brain (Whittaker, 1970) the feasibility of utilizing the synaptosome as a model for the central consequence of the proposed interaction appears highly warranted. Specifically, those synaptic events to be considered, in terms of characterization of their unique capacity to utilize a given putative transmitter molecule, will be those cholinergic and adrenergic units that are temporally and functionally linked to the stress response. It is felt that statements concerning the regional, cellular, and subcellular events which participate in synaptic transmission in terms of their contribution to the synthesis, uptake, storage, and release of molecules possessing unique neurobiological properties, may be made for stress effects, nicotine effects, as well as their interaction.

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The parameters involved with the experimental procedures will be those outlined earlier in the design and several areas of the rodent brain will be specifically considered in view of their morphological and functional interrelationship to one another. Specifically, the cerebral cortex, limbic system, and cerebral cortex will be utilized for the fractionation procedures in which synaptosomes will be prepared; further fractionation of these units will be done in order to derive cytoplasm, membrane, vesicular, and mitochondrial fractions. The details of such fractionation as well as the rationale underlying structural specificity and biochemical individuality of these systems is supported in some of the appended material.

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In general, the goal of these proposed experiments is to relate central nervous system changes, defined at the synaptic level, which occur as a consequence of acute or chronic stress and/or the interaction of such responses with nicotine. There is a further goal involving the relationship of time course for regional synaptic changes occurring at the central level with specific peripheral systems as considered earlier in this discussion.

(9) Physical Facilities Available.

Two large air-conditioned laboratories and adjoining animal housing facilities are available and currently is use by the investigator; the laboratories are completely equipped with all equipment necessary for the biochemical procedures required, with the exception of those equipment items requested on the budget.

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Supporting data and indications of project feasibility.

The appended grant progress report represents a summary of thirty-seven key experiments wherein data in support of chronic stress or chronic-acute stress interactions have been considered in view of physiological, pharmacological, neurochemical and behavioral changes observed in our laboratory with one species of mouse. There are in addition, several pending publications which have been appended to this application; these bear further upon some of the evidence cited in sections of the foregoing proposal and provide considerable support to warrant the use of both the techniques outlined as well as the experimental parameters.

Additionally, recent supporting data from our laboratory has been summarized. In Tables 1 and 2, the effects of chronic stress (isolation) are indicated with regard to the hepatic microsomal metabolism of pentobarbital and pentobarbital sleeping time; it may be observed from these tables that the stressed animals showed shorter barbiturate sleeping times and can be accounted for by an increased rate at which the drug metabolism enzyme is included in the liver. In further consideration of the differences in this regard, Table 3 illustrates the effects of chronic stress (isolation) on liver weights in mice as a function of the duration of such stress. It may be observed that as the duration of stress is increased, the liver weight is increased relative to total body weight, as compared with non-stressed animals.

In Table 4, it may be observed that increased chronic stress leads to increased in in vivo incorporation of amino acid into protein and from Table 5, it may be observed that microsomal ATPase activity is reduced both in liver and brain fractions as a consequence of isolation. Oxidative phosphorylation in liver mitochondria was generally not as great in magnitude (Table 6) as one might expect.

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In Table 7, data for in vitro protein synthesis by liver microsomes are summarized and these data confirmed previous observations indicating an increase in such synthesis as a consequence of chronic stress.

Brain protein synthesis was significantly altered in the myelin fraction from the cerebral cortex of chronically stressed mice as shown in Table 8. It should be pointed out that these observations are merely preliminary and considerably more data should be outlined before any more definite conclusions can be reached. It is clear, however, that morphological, biochemical, and cellular changes are associated with stress and may be utilized as indices in assessing such stress effects. The effects of nicotine treatment both per se, as well as in interaction with acute and chronic stress would appear ideally to constitute a relevant independent variable by which, particular cellular changes may be further studied. Inasmuch as the active constituents in tobacco smoke, aside from nicotine, warrant consideration as pharmacological variables, it would seem that those systems proposed constitute firm bases upon which such pharmacological evaluation may be approached.

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